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POSSIBLE ASSOCIATION OF THE SIGLEC-14/5 FUSION GENE WITH SEVERE MALARIA

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POSSIBLE ASSOCIATION OF THE SIGLEC-14/5 FUSION GENE WITH SEVERE MALARIA

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ABSTRACT

Nearly 225 million cases of malaria were confirmed worldwide in 2009, causing 781,000 deaths. A majority of those deaths were among children in sub-Saharan Africa according to the World Health Organization. In this project the SIGLEC14/5 fusion gene was tested for its association with severe malaria, a complicated form of the disease, to help determine whether this copy number variant (CNV) plays a role in the genetic risk of severe malaria. Matched malarial cases and controls from Tanzania were genotyped using conventional PCR methods, then the data was compiled. The results indicate there is a possible association between this fusion gene and severe malaria. Further studies will need to be completed to confirm these assumptions.

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BACKGROUND

Malaria

Nearly 225 million cases of malaria were confirmed worldwide in 2009, causing 781,000 deaths. A majority of those deaths were among children in Africa, resulting in a child's death every 45 seconds due to malaria (WHO, 2010). Malaria is caused by the parasite *Plasmodium falciparum*, and its global distribution is shown in **Figure 1**. Most of the cases (green) are concentrated in Africa, with significant numbers in Asia and South America. It is estimated that about 70% of malarial cases occur in Africa and about 25% in South East Asia (Snow et al., 2005).

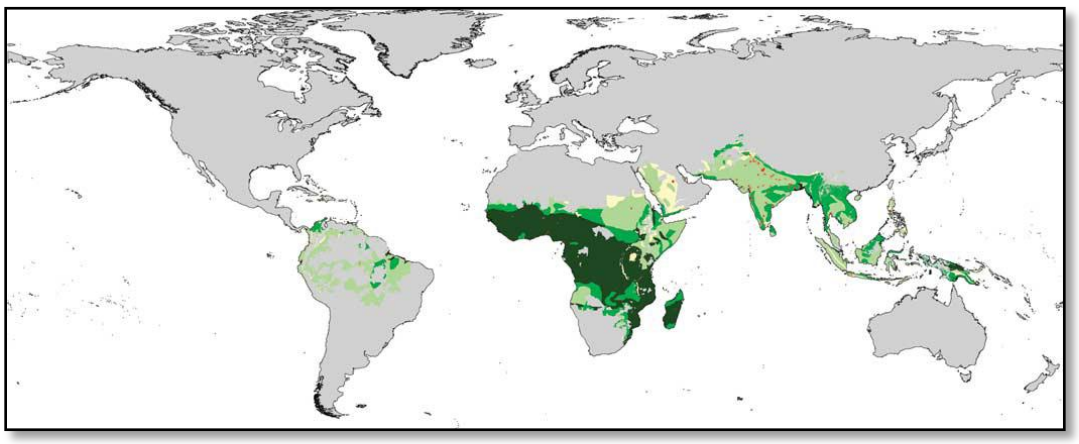

Figure 1: Global Distribution of *P. falciparum* Endemicity. (Snow et al., 2005).

Of the hundreds of *Plasmodium* species, only four are known to cause human malaria. *Plasmodium falciparum* and *Plasmodium vivax* are the most common species. *P. vivax* is likely the most widespread malarial parasite due to the large populations within Asia, Latin America, and parts of Africa that it inhabits. The deadliest, however, is *P.*

falciparum causing most of the deaths in Africa south of the Sahara, as well as Papa New Guinea. Because this species multiplies quickly within the blood of its host, it can cause the onset of severe anemia. Cerebral malaria, hypoglycemia, respiratory distress and metabolic acidosis are also strictly associated with *P. falciparum* (CDC, 2010).

Climate is a main factor for the transmission of malaria. Temperature, humidity, and rainfall all influence the survival and reproduction of the *Anopheles* mosquitoes that act as a vector for the spread of the parasite. The climate also affects the parasite's ability to complete its life cycle within both the vector and host. At temperatures below 20°C, *P. falciparum* is unable to complete its life cycle. Thus, malaria is typically transmitted in tropical and subtropical areas where the conditions are more favorable. Even within these areas, other variables are unfavorable for transmission such as higher altitudes, colder seasons, and deserts where there is a lack of water for which the mosquitoes breed. The highest transmission of malaria occurs in regions around the equator where temperatures are much warmer (CDC, 2010).

Life Cycle of *P. falciparum*

When a female mosquito infected with *P. falciparum* takes a blood meal from a human host, sporozoites of the parasite are injected into that host. These sporozoites then infect the liver cells where they mature into schizonts that release merozoites when they rupture. The parasites undergo asexual reproduction in the erythrocytes after their initial replication in the liver cells. The merozoites then infect the red blood cells where they can either differentiate into gametocytes or continue the cycle of producing more

merozoites through the maturation of schizonts. These blood stage parasites are responsible for the appearance of the symptoms of malaria (CDC, 2010).

Those merozoites that differentiate into gametocytes can be ingested by a female mosquito during a blood meal of an infected individual where the parasite reproduces during the sporogonic cycle forming zygotes. These zygotes are elongated and motile allowing them to invade the midgut wall of the mosquito. Here they develop into oocysts that mature and rupture to release sporozoites that are able to be injected into the host through the mosquito's salivary glands. This occurs 10-18 days after the gametocytes were ingested into the mosquito. This cycle enables the mosquito to pass the parasite on to multiple hosts causing widespread infection with malaria (CDC, 2010).

Malaria Diagnosis and Treatment

The symptoms of malaria can be mild or severe. Severe malaria results from *P. falciparum* infection with delayed treatment (WHO, 2000). "Severe malaria occurs when infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism (CDC, 2010)." Within the sub-Saharan Africa regions, there are three different manifestations of severe malaria which include cerebral malaria, acute respiratory distress, and severe malaria anemia which occur predominately in children (Bejon et al., 2007). Typically, patients are misdiagnosed due to the common symptoms such as fever, diarrhea, headache, and body aches and pains. Patients lacking proper treatment tend to deteriorate quickly and experience a wide range of symptoms that may occur in combination with one another or individually (WHO, 2000).

Antimalarial drugs have been developed. One of the most common drugs used to treat severe malaria is Quinine given intravenously via infusion pump. Other antimalarial drugs include Artemisinin**,** Artesunate, Artemether, Sulfadoxine–pyrimethamine, and Chloroquine (WHO, 2000). During the mid-1900's Chloroquine was the preferred antimalarial drug, however due to its wide use resistance against the drug grew quickly primarily in Southeast Asia and South America. These regions have abandoned use of Chloroquine, but because of its affordability many countries in Africa still use it as their main defense against malaria. As resistance rates increase, this drug is becoming less of an option in all parts of the world (Djimde et al., 2001). Mefloquine is another antimalarial drug that is effective against falciparum malaria. This drug has particular importance because within 6 years of its release in 1984 significant resistance had been developed (Price et al., 2004). Currently, artemisinin-based combination therapy (ACT) is the most effective treatment available (WHO, 2010).

Malaria and Copy Number Variants

Alterations of genomic DNA that span more than 1000 bases are known as structural genetic variation. The most prevalent type of structural genetic variation is copy number variants (CNVs). CNVs have been known to cause genomic disorders including Williams–Beuren Syndrome and Charcot–Marie Tooth neuropathy Type 1A in which there are repeated CNVs at critical loci. Additionally, CNVs are present throughout the human genome among otherwise healthy individuals. Keeping this in mind, research is currently being done to identify how CNVs may influence more common human diseases (Ionita-Laza, et al., 2009).

There are several features of CNVs that sustain their part in disease pathogenesis. First, the large size of CNVs disrupts sequences of functional DNA causing the cell to behave abnormally. Second, large quantities of CNVs seem to be concentrated in genes that help humans to adapt to changes in their environment, such as those genes that code for the immune system. Third, CNVs appear to be influenced by natural selective pressures indicating that their functions are significant. Lastly, "53% of genes whose expression was influenced by CNVs had the corresponding CNV outside of the actual gene, suggesting that many CNVs could affect important regulatory sequences that are situated at a distance from the actual target gene (Ionita-Laza, et al., 2009)." **Table 1** lists several diseases associated with CNVs.

Table 1: CNVs Associated with Complex Genetic Disease.

CNV	Complex Disease Associations
CCL3L1	HIV/AIDS susceptibility caused by decreased copies (Gonzalez, et al., 2005). Increased risk of rheumatoid arthritis due to increased copies (McKinney et al., 2007).
FCGR3B	Increased risk for lupus nephritis due to decreased copies (Aitman et al., 2006).
UGTB17	2-fold increased risk of osteoporosis associated with deletion (Yang et al., 2008).
C4	Increased risk of systemic lupus erythematous associated with deletion (Yang et al., 2007).
DEFB4	1.7-fold increased risk of psoriasis associated with >5 copies of beta-defensins (Hollox et al., 2007). 3-fold increased risk of Crohn disease associated with <4 copies (Fellermann et al., 2006).
GSTM1	Allergic responses, impaired lung function, and asthma associated with deletion (Brasch-Anderson et. al, 2004).
LCE3B & LCE3C	Psoriasis associated with multigene deletion of late cornified envelope genes (de Cid et al., 2009).

With respect to malaria, in a population in West Africa it was shown that individuals having the HH131 genotype show increased susceptibility to severe malaria. A majority of these individuals were children less than five years old, an age group in which most cases of severe malaria occur. This genotype was caused by a copy number variant on the Fc receptor, FcγRIIa, in which a single nucleotide change alters a histidine (H) to an arginine (R) residue at position 131 and creates function change. This molecule provides a link between the humoral and cellular immune systems through its expression on the surface of lymphocytes and monocytes. This association between CNVs and

severe malaria provides information about the immune system's capability of protecting individuals from severe malaria (Cooke et al., 2003).

Siglecs

Glycan-recognizing proteins within animals are classified into two groups which include lectins and sulfated glycosaminoglycan (SGAG)-binding proteins. I-type lectins are those proteins that mediate the identification of glycans through their immunoglobulin (Ig)-like domains. Siglecs (*S*ia-recognizing *Ig*-superfamily *lec*tins) are a subfamily of these I-type lectins having sialic acid (Sia)-binding properties and amino-terminal structural features. One subgroup of the siglecs are those that have been conserved throughout evolution which include Siglecs-1, -2, and -4. The other is a CD33/Siglec-3-related subgroup which includes Siglecs-3 and -5–13 in primates that has been quickly evolving (Varki and Angata, 2006). For the purposes of this project, the siglecs will be the main focus.

Figure 2 depicts the main structural features of siglecs pertinent to their functions. The N-terminal 'V-set' Ig domain of each siglec (A in the figure) binds ligands containing sialic acid. Each siglec also has anywhere from one to sixteen 'C2-set' Ig domains (B in the figure) lengthen the distance between the ligand binding site and the membrane surface. Specificity for sialoside sequences on glycoprotein and glycolipid glycans present on either the same cell or neighboring cells by each siglec is variable. Immuno-receptor tyrosine-based inhibitory motif (ITIM) and ITIM-like motifs (C in the figure) are contained within the cytoplasmic domains of CD22 and most CD33-related siglecs.

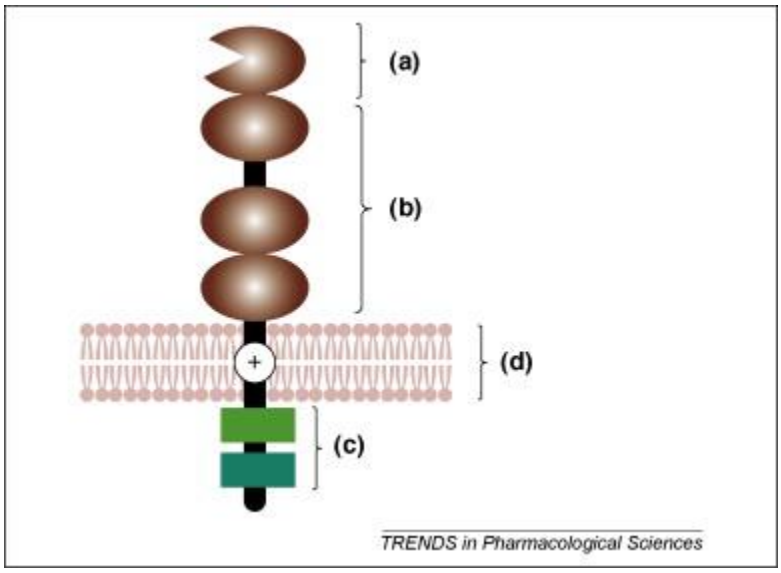

Figure 2: Common Structural Features of Siglecs. The N-terminal 'V-set' Ig domain **(a)** contains a conserved arginine residue that confers sialic-acid-binding ability. This domain is followed by a variable number (1–16) of 'C2-set' Ig domains **(b)**. In the cytosolic domain, most siglecs contain some combination of tyrosine motifs including ITIM, ITIM-like, Grb2-binding and Fyn kinase sites **(c)**. Siglec-14, Siglec-15 and Siglec-16 contain a positively charged residue in the transmembrane spanning region **(d)** that enables association with the ITAM-bearing adaptor protein DAP-12. It is speculated that these might have evolved to counteract ITIM-bearing siglecs. With 99% sequence identity in the two first N-terminal Ig domains, Siglecs-5 and Siglecs-14 are believed to be paired receptors (O'Reilly and Paulson, 2009).

The regulation of cell signaling is mediated via these Siglec motifs. However, Siglec-14 to Siglec-16 and murine Siglec-H do not contain tyrosine motifs but rather have a transmembrane-spanning region that is positively charged. This positive charge allows for the adaptor protein DAP12 to associate with the charged region. DAP 12 contains a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) which can transmit both positive and negative signals (O'Reilly and Paulson, 2009). DAP12 is abundantly expressed in "plasmacytoid dendritic cells, dendritic cells, neutrophils, basophils, eosinophils, monocytes, macrophages, microglial cells, and osteoclasts, as well as natural killer (NK) cells (Lanier, 2009)." Ligands encoded by either the host or by microbial pathogens have been shown to be recognized by DAP12-associated receptors.

This suggests that DAP12 plays an important role in initiating innate immune responses. Typically, when receptors employing ITAMs as modes of signaling, such as DAP12, are ligated they initiate cellular activation. Cytokines are then produced which causes degranulation in cytolytic effector cells (Lanier, 2009).

Most CD33rSiglecs have formed a gene cluster located on the human chromosome 19 indicating that they have evolved substantially through gene duplications, deletions, and chimerism (Angata et al., 2006). Siglec-5 and Siglec-14 have extensive sequence similarity and are proposed to be paired inhibitory and activating receptors. Via its arginine reside in the transmembrane domain, Siglec-14 can associate with the signaling adaptor molecule DAP12 which suggests that it functions as an activating receptor (Yamanaka et al., 2009). Siglec-5 has been found to be expressed on monocytes, granulocytes, plasmacytoid dendritic cells, monocyte-derived dendritic cells, and macrophages. Siglec-5 is also "potentially involved in the negative regulation of innate immune responses (Angata et al., 2006)." In Yamanaka et al., a copy number variation was found between the region of the SIGLEC14 and SIGLEC5 genes fusing the two genes into what is called a SIGLEC14/5 fusion gene whose function is not fully understood. In this project, the possible association of this SIGLEC14/5 fusion gene with severe malaria will be investigated.

Previous Work

Before beginning this project, the Bailey lab had identified Siglec-14 and Siglec-5 genes as possible candidates for correlation with the contraction of severe malaria during an investigation of copy number variation via array comparative genomic hybridization

(CGH) of severe malaria cases and controls. To validate the hybridization analysis, Dr. Reyburn provided the Bailey lab with 504 blood samples of severe malaria and 504 village-matched controls, to be used in determining the effect that copy number variations may have on the contraction of severe malaria. For this project these samples were used to evaluate the possible association of the SIGLEC14/5 fusion gene with severe malaria.

PROJECT PURPOSE

In this project, the SIGLEC14/5 fusion gene will be tested for its association with severe malaria. This analysis will help determine whether CNVs play a role in the genetic risk in severe malaria. To do this, DNA was isolated from provided blood samples, and genotyped using conventional PCR methods. The number of cases having the 14/5 fusion gene compared with the number of controls having the fusion gene was then assessed. Based on the significance of the results of this genotyping, the association of the fusion gene with severe malaria can determined. Following this project, future studies will need to be completed to confirm the results. Possibly, this information can then be used to develop drug therapies that guard against severe malaria.

METHODS

PCR Design

In the Yamanaka et al. (2009) study, the parameters for determining the genotype of individuals with regards to SIGLEC 14, SIGLEC 5, and SIGLEC 14/5 fusion genotypes were established. **Figure 3** depicts the primers and possible genotyping outcomes from this study. Wild type individuals have both SIGLEC 14 and SIGLEC 5 genes intact, while null individuals have the SIGLEC 14/5 fusion gene, a deletion of a segment of DNA between SIGLEC 14 and SIGLEC 5 fusing the two genes. These genes were previously recognized by the Bailey lab as possible candidates for correlation with the contraction of severe malaria by a comparative genomic hybridization study.

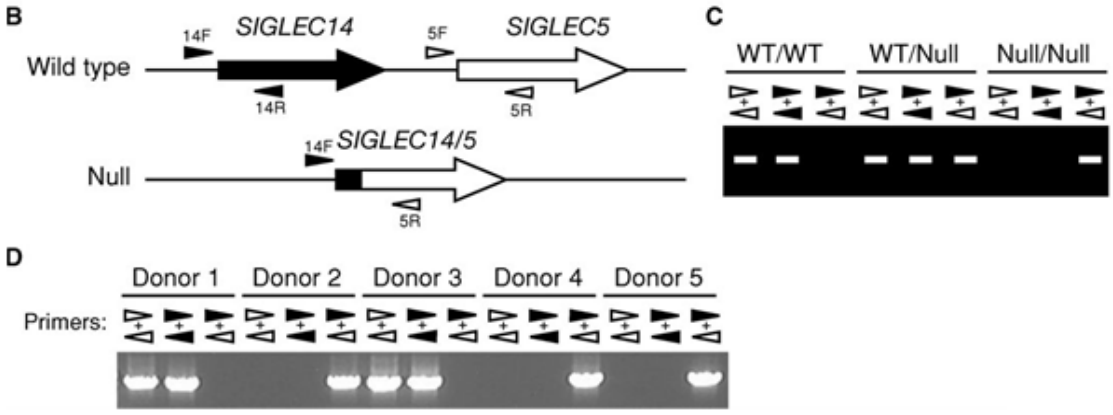

Figure 3: (B) Schematic Diagram for Genomic PCR of Siglec 14/5. The primer pair 5F + 5R specifically amplifies a part of the SIGLEC5 gene, and the primer pair 14F + 14R a part of the SIGLEC14 gene. Genomic PCR with the primer pair 14F + 5R yields a ~1.6 kb product only when the SIGLEC14/5 fusion gene is present. This pair can theoretically amplify a ~17 kb fragment from the wild-type allele as well, but failed to do so under the conditions used. (C) Expected gel electrophoresis pattern of PCR products for each genotype. (D) Genomic PCR results of the same set of individuals as in A. Donors 2, 4, and 5 are homozygotes for the null allele, as expected. (Yamanaka et al., 2009)

Blood Samples

Samples of whole blood were provided from collection sites in Tanga, Tanzania, in East Africa (Dr. Reyburn, KCMC Joint Malaria Program). Dr. Reyburn's study consisted of 504 hosptial malarial cases and 504 village controls. All cases and controls were matched based on age, ethnicity, and season by the collaborators at these sites. Dr. Reyburn is continually collecting samples providing potential new cases and controls to use in the future. In addition, a site in Papua New Guinea (Dr. Mueller, PNG Institute of Medical Research) is currently completing a series of 400 cases and 400 controls of severe malaria that will provide us in the future with additional samples to continue further testing. Both studies have ensured that samples are truly taken from severe malaria patients by conducting blood cultures. Those cultures shown to contain bacterial infections associated with asymptomatic parasitemias are confirmed as severe malaria cases and deemed useable. Ethical approval was received to conduct all of these human genetic studies. The whole blood samples (biosafety level BSL-2) were handled by other personnel in the lab trained at BSL-2 levels.

DNA Isolation

DNA was isolated from whole blood samples by other personnel in the lab trained at BSL-2 biosafety levels using Qiagen's Blood & Cell Culture DNA Kit. Purified DNA samples were no longer BSL-2 so could easily be handled for genotyping experiments.

Siglec Genotyping

In an attempt to validate and extend the information presented in the Yamanaka et al. (2009) study, a triplet set of primers were developed similar to their design, and were

used to genotype 362 samples. However, those primers failed to show amplicons of the expected sizes, so a set of new primers were developed. Primers used for the specific amplification of SIGLEC 14, SIGLEC 5, and SIGLEC 14/5 fusion genes were as follows: Sig F-24098a (5'-CCTACCTATGCCGTTCCTGA-3'), Sig R-25669a (5'-AGCTGGACAAGCCTCACATC-3'), Sig R-del25970a (5'-GGGGACCACATCTCTCTTGA-3'). These primers were designed using the Primer 3 website and manufactured by Invitrogen. The primer triplets were used in amplification of genes homozygous for the SIGLEC 14/5 fusion product (Null/Null), homozygous for the SIGLEC 14 and SIGLEC 5 (WT/WT), or heterozygous for SIGLEC 14 and 5 and the SIGLEC 14/5 fusion product (WT/Null).

Each PCR reaction tube contained the following for a total of 20 µL: 2 µL genomic DNA (~10 ng), 2 µL 10X PCR buffer, 0.4 µL dNTP solution (10 mM), 0.2 µL Platinum® Taq DNA Polymerase, 0.6 µL Magnesium Chloride (50 mM), 0.2 µL of each primer listed above, and 14.2 µL Sterile H_2O. Thermal cycling parameters were as follows: 94°C for 2 minutes; (94°C for 20 seconds, 56°C for 30 seconds, 72°C for 100 seconds) × 30 cycles; 72°C for 7 minutes; hold at 4°C. Completed PCR samples were then prepared to run on agarose gels by combining the following in each new tube: 3 µL Bromophenol blue dye and 8 µL PCR product. These were electrophoresed along with a standard DNA ladder. The agarose gel was prepared by combining the following: 0.6 g agarose, 50 mL 1X TAE Buffer, 1 uL ethidium bromide. **Figure 4** depicts the new primers and possible genotyping outcomes.

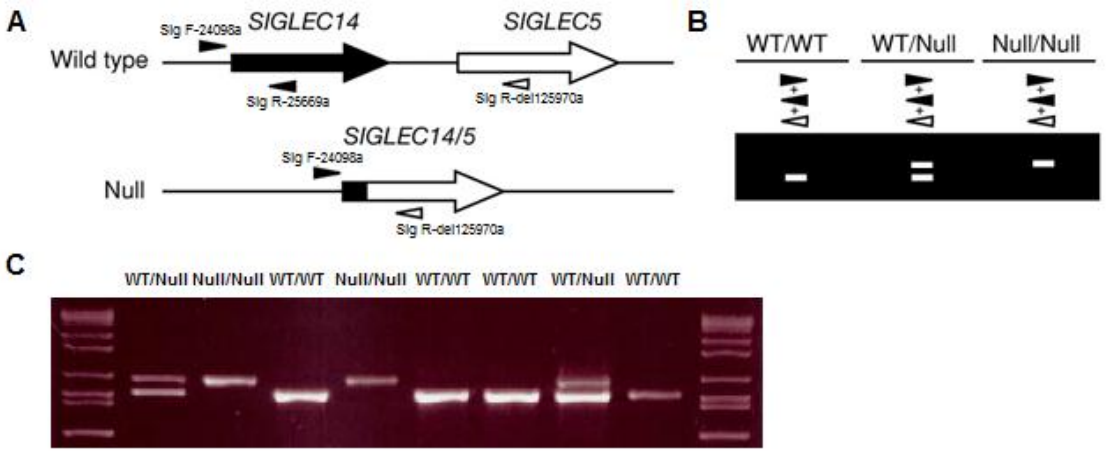

Figure 4: Siglec Primers Used in this Study and Genotyping Outcomes. (A) The primer pair Sig F-24098a and Sig R-25669a amplifies a portion of the SIGLEC 14 gene, if present, and produces the lower PCR product band seen in (B) and (C). The primer pair Sig F-24098a and Sig R-del125970a amplifies a portion of the SIGLEC 14/5 fusion gene, if present, and produces the upper PCR product band in (B) and (C). (B) Expected gel electrophoresis pattern of PCR products for each genotype. (C) Genomic PCR results of eight example individuals with their corresponding genotype.

RESULTS

To determine whether the SIGLEC14/5 fusion gene is associated with severe malaria, 362 blood DNA samples were genotyped using conventional PCR. The results were compiled in the following tables displaying the genotype for each malarial case and matched controls. These tables also include statistical data including Chi-square values, p-values, Yates' Chi-square values, and Yates' p-values. Chi-square tests are used to detect group differences using frequency data. The Chi-square value and the degrees of freedom were used to calculate a p-value. Calculating the p-value can help determine if a random sampling from the data is likely to lead to a significant difference in the observed results. Typically, when the p-value is less than 0.05, the results are thought to be statistically significant.

Table 2 shows the total set of 362 unmatched samples that were genotyped. The number of individuals with severe malaria and the WT/WT genotype was about the same as the number of individuals without severe malaria and the WT/WT genotype. Likewise, the number of individuals with severe malaria and the WT/Null genotype was about the same as the number of individuals without severe malaria and the WT/Null genotype. Again, the number of individuals with severe malaria and the Null/Null genotype was about the same as the number of individuals without severe malaria and the Null/Null genotype. These results produced a p-value of 0.196 meaning that they are not statistically significant. From this data there does not seem to be any relationship between the SIGLEC14/5 fusion gene and resistance to severe malaria. However, keep in mind that the samples shown in Table 2 are unmatched, meaning that differences in age,

ethnicity, and season were not accounted for and could have caused the data to be skewed.

Table 2: Genotyping Results for 362 Unmatched Samples.

Genotyping Results of Total Data Set (362 Unmatched Samples)			
Genotype	**Case**	**Control**	**Total**
WT/WT	89	82	171
WT/Null	69	85	154
Null/Null	22	15	37
Total	**180**	**182**	**362**

Chi-square value:	3.262
Degrees of Freedom:	2
p-value:	0.196
Yates' Chi-square:	2.623
Yates' p-value:	0.269

In the second set of results (**Table 3**) the statistical analysis was restricted to only include genotypic data from samples where data from both the severe malarial case and the matched control were available. In this case, for the WT/WT Siglec genotype, the number of individuals with severe malaria is approximately equal to the number of control samples. This is also the case for the WT/Null genotype. However, for the Null/Null genotype, the number of severe malarial cases is more than double the control

cases. These results produced a p-value of 0.031 meaning that they are statistically significant. From this data it seems that there is likely a relationship between the SIGLEC14/5 fusion gene and increased susceptibility to severe malaria.

Table 3: Genotyping Results for 228 Matched Samples.

Genotyping Results of All Matched Cases and Controls (228 Matched Samples)			
Genotype	**Case**	**Control**	**Total**
WT/WT	54	57	111
WT/Null	42	51	93
Null/Null	18	6	24
Total	**114**	**114**	**228**

Chi-square value:	6.952
Degrees of Freedom:	2
p-value:	0.0310
Yates' Chi-square:	5.766
Yates' p-value:	0.0560

DISCUSSION

From this study it was determined that data from the entire 362 unmatched sample set (Table 2) produced p-values well above 0.05, indicating that no association appeared to exist between severe malaria and the Siglec 15/5 fusion genotype in the total set of unmatched samples, but when the data set was restricted to a subset of 228 matched samples (Table 3), the resulting p-values suggest that an association may, in fact, be valid. This indicates that the fusion genotype may correlate with an increased susceptibility to severe malaria.

During this project few problems were encountered, but the few problems that did exist were time consuming to solve. First, the primers used in the earlier Yamanaka study proved to be unsuccessful in producing any amplicon product for the blood DNA samples that were used in this project. So a new set of primers had to be developed and tested for productivity. This set of primers eliminated the need for both a primer set for SIGLEC14 and another primer set for SIGLEC5 for which multiple PCR reactions would have been required. Instead, the new triplet set of primers provided product for each genotype within a single PCR reaction. Second, the parameters of the PCR reaction had to be adjusted to produce adequate readings of the results. The time for initial denaturation had to be extended and the annealing temperature was adjusted slightly. For the denaturation, annealing, and extension period, the time had to be increased significantly while adjusting the temperature slightly. The number of cycles that this period completed was increased in an attempt to produce more product. The final extension time had to be decreased as well. Finally, the type of Taq polymerase was changed from that found in

the Bio-Rad iProof™ High-Fidelity PCR Kit to the Platinum® Taq DNA Polymerase provided by Invitrogen.

Copy number variations within the human genome have previously been found to be associated with immunity and infectious disease. In this project it is believed that the SIGLEC14/5 fusion gene may provide individuals with resistance to severe malaria similar to the CNV found on FcγRIIa that caused increased susceptibility to severe malaria in the Cooke study. Although not much is known about the SIGLEC14/5 fusion gene and its functions, this project presents information from which assumptions can be made. Perhaps the normal function of the Siglec-14 and Siglec-5 genes is to associate with the adaptor protein DAP12 causing activation of natural killer cells that can then destroy invading *P. falciparum* parasites. This fusion gene may disrupt this function leaving cells vulnerable to invasion.

This project was successful in determining that there is a possible association between the SIGLEC14/5 fusion gene and severe malaria. However, continued genotyping of the samples provided by Dr. Reyburn's study would provide a larger sample size making for more reliable data. Determining the tribe within Tanzania from which each sample was taken would be helpful in determining if certain data sets should be excluded from the study. This data might also be useful in tracking the genetic lineage of this gene throughout that region. Given that the fusion gene proves to be strongly associated to severe malaria, future studies into the possible development of drug therapies could be a viable option to help combat severe malaria.

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